DNA PROBES FOR THE IDENTIFICATION OF MICROBES

Wayne P. Olson
Consultant, Beecher, Illinois, U.S.A.

INTRODUCTION AND SCOPE

In 1997 and the first quarter of 1998, when much of the first version of this article was written (1), what we hereafter refer to as DNA probes were used primarily by those performing genetic-related research. Today (mid-2000), oligonucleotide (DNA, RNA, and related) probes in several guises are a growth industry in biotechnology, and their applications are widespread. In 1995, Theta Reports (*Gene Therapy/DNA Probes/PCR Markets*) predicted a phenomenal growth in polymerase chain reaction (PCR), DNA probes, and the successful use of gene therapy—they were correct about the first two. In 1996, the Frost & Sullivan Market Intelligence indicated that the 1995 market for DNA probes had been over $145 million, and predicted that it would reach $1.4 billion by 2003 (*U.S. DNA Probe Markets*). Also in 1996, Business Communications Co., Inc., made similar predictions (*Advances and Opportunities in DNA Testing and Gene Probes*). DNA probes are the fastest growing area of in vitro diagnostics (2), expanding at the rate of 25% per year.

The most important differences between the earlier and the current version of DNA probes (1) relate to the following:

- Literature and applications have boomed; cumulative citations listed by Medline for "DNA probes" are currently 79,270.
- Applications—especially of PCR—now embrace most aspects of life sciences, including agriculture, food science, and nonmicrobial disease states, as well as areas relating to human diseases.
- Use of oligo-on-a-chip (DNA- or RNA-based array, also referred to in some instances as microchip) has burgeoned in pharmaceutical/biotech R&D. This approach, if not yet predominant, is becoming so in many applications.
- Antisense therapeutics, which had lost favor due to a well-publicized Phase-I human death in mid-2000, are already rebounding. Some forms of antisense show very high potential as antimicrobials.

To focus the current version of "DNA probes" on microbiological and related issues, together with literature not explored in the previous version (1) some background material has been abbreviated. The reader unfamiliar with the fundamentals of DNA probes is urged to consult Vol. 19, Ref. 1 for introductory particulars. Table 1 of that article is repeated herein as Table 1, but without references. This author also recommends that the genomics tyro read one or more of the basic references (3–5), of which the article by Keller and Marak is the most germane. Additional basic texts not cited in (1) are listed in Table 1. Editors of those texts, who are significant contributors to the scientific literature, include (to my knowledge) Clapp, Higgins, Kricka, Marak, and Persing. Presumably, the others also do fundamental research but I have not read their papers.

Rapid growth areas of science, such as oligo probes, are referenced most rapidly on the Internet. Formal print publication may appear 1–3 years after completion of the work. Many, but not all, Internet publications are refereed in a manner similar to that for print publications. Therefore, many of the references herein are from the Internet.

Although I have cited a very small proportion of the patent, regulatory, and market literature herein, the reader should note that

1. Many productive scientists choose to file for letters patent in lieu of, rather than in addition to, conventional publication.
2. Negative findings may not find their way into scientific literature for a long time, if ever.
3. For a new commercial venture, first-rate technology is often insufficient; good business practice and adequate financing are equally important. Therefore, many small operations disappear.

Follow-up on vanished companies and products is difficult, unless they are acquired. For example, Biotech Research Laboratories and their peptide nucleic acid oligos (PNAs) were acquired by Boston Biomedica, Inc. Tracking BRL and its specifically targeted PNAs to BBI is simplified by web-page links, which are easy to follow for the reader with a modest knowledge of computer use.

Table 1 Texts entirely, or in part, about oligonucleotide probes

Year	Editor(s)	Title	Publisher
1996	Persing, D.H.	*PCR Protocols for Emerging Infectious Diseases*	ASM Press
	Clapp, J.P.	*Species Diagnostic Protocols: PCR and Other Nucleic Acid Methods, Vol. 60*	Humana
1995	Hames, B.D.; Higgins, S.J.	*Gene Probes: A Practical Approach, Vols. 1 & 2*	Oxford University Press
1994	Svendsen, P.; Hau, J.	*Handbook of Lab Animal Science, Vols. 1 & 2*	CRC Press
1993	Keller, G.H.; Manack. M.M.	*DNA Probes*	Stockton Press
1993	Persing, D.H.; White, T.J.; Tenover, F.C.; Smith, T.F.	*Diagnostic Molecular Microbiology: Principles and Applications*	ASM Press
1991	Stanley, P.E.; Kricka, L.E.	*Bioluminescence and Chemiluminescence: Current Status*	Wiley
1990	Boulton, A.A.; Baker, G.B.; Campagnoni, A.T.	*Molecular Neurobiological Techniques*	Humana

DEFINITIONS

Amplification of an oligonucleotide or an entire gene: See polymerase chain reaction.

cDNA: Copy DNA, as by PCR or RT-PCR.

DNA probe: An oligonucleotide sequence that complements a sequence (usually of a gene) in or from an organism. Here, DNA is used generically in the description of probes.

dsDNA: Double-stranded DNA, as in the double helix.
endonuclease: An enzyme that hydrolyzes (breaks) an oligonucleotide chain between particular bases only; consequently, the pattern of pieces of DNA (oligos) generated by the action of endonuclease on DNA of a particular organism is reproducible.

Genome: The entire DNA complement [gene(s)] of an organism; this includes the genetic information in viruses.

Morpholino oligo: An oligo containing the morpholino analog of the conventional backbone (Fig. 1a).

Table 2 Commercial firms producing and/or selling oligonucleotide probes and arrays (partial listing, in alphabetical order)

Probes	Affymetrix
Bayer	Aventis
Chiron	BioMerieux
Digene	Nanogen
Gen-Probe	Oncogene Science Diagnsotics
Roche	Roche Molecular Systems
Oliogos-on-a-chip	

Nucleoside: Molecule containing a purine or a pyrimidine base linked to a pentose sugar.

Nucleotide: A phosphorylated nucleoside.

Oligonucleotide (also termed oligos): Usually a sequence of DNA or RNA with a phosphate backbone (Fig. 1b), but may have a sulfate, peptide, or morpholino backbone in place of a phosphate one, to reduce or eliminate oligo degradation by nucleases.

PCR: See polymerase chain reaction.

Phosphorothioate oligo: An oligo (usually a probe) containing S in place of P (Fig. 1c).

PNA oligomer: Peptide nucleic acid oligo (Fig. 1d); manufacturers include Boston Probes (www.bostonprobes.com); the Danish company; Pantheco (www.pantheco.com), and Research Genetics (www.resgen.com). These, like morpholino analogs, resist nuclease digestion.

Polymerase chain reaction: The method for producing, in vitro and fairly rapidly, millions of copies of a specific segment of DNA or RNA (amplification). The sequence of the ends of the portion to be copied must be known so that the primer can be annealed to the denatured oligo to be copied. An excellent source for PCR applications is www.eppendorfsi.com/application.html.

Primer: The segment of synthetic oligo that is added to one end of the DNA or RNA to be copied. The denatured oligo target is annealed with the primer, and DNA polymerase then adds complementary nucleic acids until the full copy is produced. See Ref. 1 for figures and further explanation.

Reverse transcriptase (RT): The enzyme from RNA viruses that codes (cDNA) from RNA.

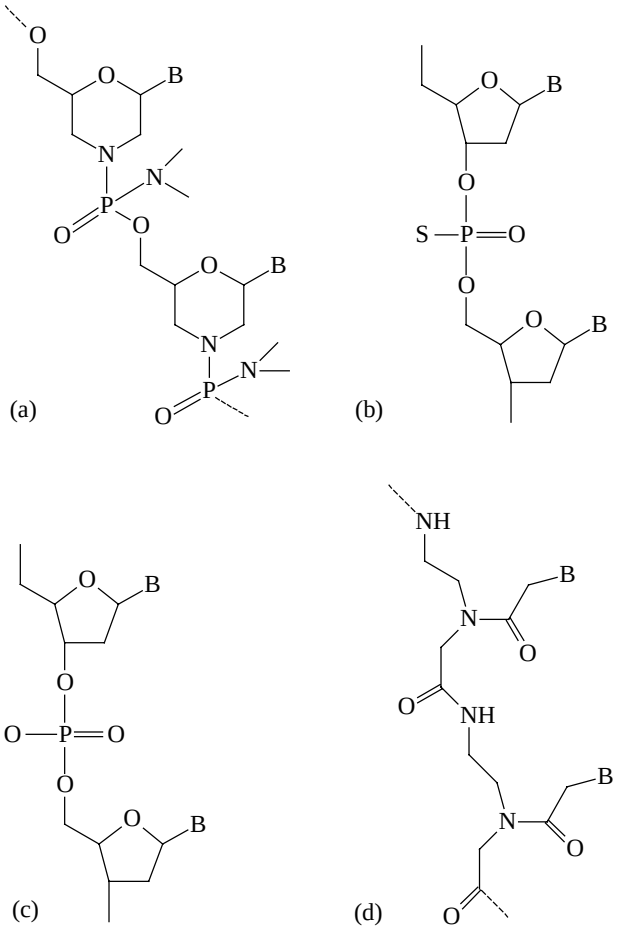

Fig. 1 Dinucleotide structures (as found in respective dinocleotides). (a) morpholino analog; (b) normal DNA or RNA; (c) phosphorothioate analog of DNA; (d) peptide nucleic acid.

Sense strand: In dsDNA, the strand that codes for the protein that is generated from it. The other strand is complementary, and protects the sense strand.

ssDNA: Single-stranded DNA, as contrasted with dsDNA

Target of an oligonucleotide probe: Usually ribosomal RNA (rRNA), which (with uridine in place of thymine) is the complement to the sense strand of the dsDNA in the double helix.

Triplet code: Although the coding for the amino acid sequence of proteins is by sets of three nucleotides in the DNA, the actual blueprint for protein sythesis is in RNA transcribed from the DNA. For example, in the RNA copied from a gene, UAU (uracil–adenine–uracil) *or* UAC (C is cytidine in the second uracil) are triplet codes for tyrosine.

ABBREVIATED BACKGROUND

Every characteristic of an organism (single-celled or multicelled) is dictated by its genome. For example, all metabolic processes are determined by enzymes that are encoded in the genome (some enzymes are imported and/or exported from a microbial cell in plasmids, which are small closed DNA loops of genes separate from the microbial chromosome). In some viruses, the genome is ssDNA or RNA, but in the majority of organisms, including microbes, it is dsDNA. The double helix of dsDNA contains a sense strand, which carries the sequence of the nucleotide triplet codes that characterize the amino acid sequence of the protein they code for. The other nucleotide strand in dsDNA is complementary to the sense strand and stabilizes it (Fig. 2).

Although some segments of the DNA in a microbial cell provide information that can identify the cell to species, there is only a single copy of DNA per cell. There may be 10^4 or more copies of the 16S rRNA per cell involved in the production of various proteins (usually enzymes) for use by the cell. The rRNA of molecular weight 16S (designated as Svedberg units in the ultracentrifuge) provides regions that are suitable for identification of the microbe to species (Fig. 3).

Therefore, if the 16S rRNA of an organism can be made available to oligo probes, usually by opening the organism in some way, it is possible to see whether the rRNA of the microbe anneals (forms a duplex) with the labeled synthetic oligo probe of known sequence and identity. There are several ways of doing this. However, in my view, unless the genus of the organism is known with reasonably good probability, this method is inordinately expensive in the use of labeled probes.

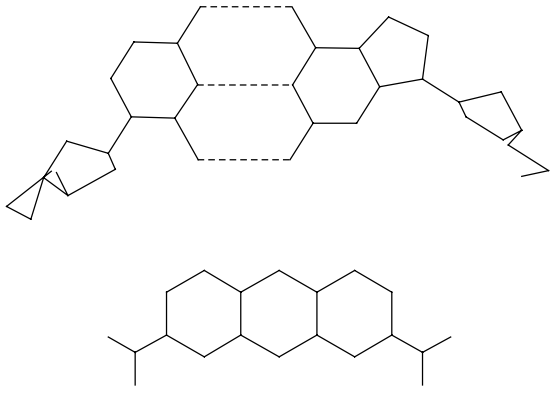

Fig. 2 Schematic of base pair in dsDNA (above) and acridine orange (AO) (below). The AO intercalates between sequential base pairs, extending the phosphate backbone.

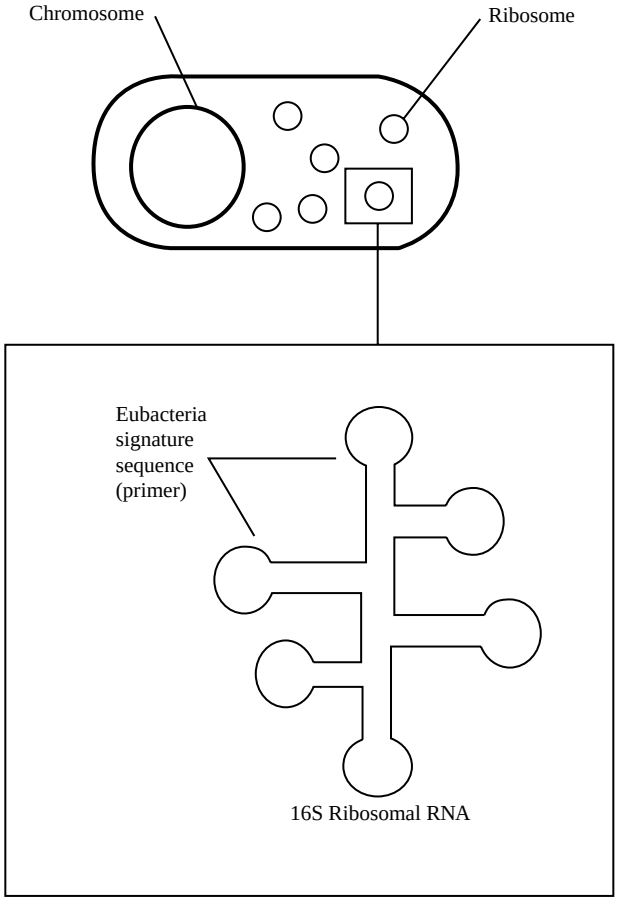

Fig. 3 Extrachromosomal ribosomes in a bacterium or fungus that contain 16S rRNA.

APPROPRIATE USE OF OLIGO PROBES

In my view, before labeled probes are put to use for the identification of an unknown, the organism should be tentatively identified. Conventional (but effective) hand methods derived from *Bergey's Manual* can be painfully slow. The fatty acid methyl ester (FAME), technique reference (6), or one of the methods outlined in (7)are usually quite accurate and sufficiently rapid if the patient is not in extremis. Also, it is wise to remember that conventional bench methods (e.g., Bergey's) are dendritic, and for microbiologists lacking considerable experience, an error in any of the sequential steps may result in error in the identification. The earlier a mistake is made in a dendritic pattern, the greater the error in identification (i.e., if a bird's nest is near the end of a limb on the north side of the tree and one chooses to climb on a limb on the south side of the tree in search of the nest, one will be right about the nest being above ground, but remote from the objective).

If preliminary information is available (e.g., indicators of pathogenicity), the most cost-effective and reliable approach may be to go directly to probes. Also, probes make best sense in widespread screening for specific diseases such as tuberculosis. In disease states, whether in humans, animals, or plants, symptoms may indicate the presence of one or a few pathogens. In such instances, the use of probes directly is sensible and economical. Machine identifications, based not on genomic but on chemical analyses, become confirming and often unnecessary.

Probes, depending on the oligo target in the organism, are for practical and definitive purposes. Errors with nongenomic machines (e.g., FAME profile by GC, or mass spectrometry/fuzzy logic profile, etc.) are because the organism sought is not in the machine library, or the concentration of key chemicals (almost always oligonucleotides, oligosaccharides, and/or FAME from the organism) is too low (insufficient cells or incomplete extraction).

For some very slow-growing organisms such as *Mycobacterium tuberculosis* and *M. leprae*, sufficient organisms may be present for analysis by probe in the sample fluid or tissue, in which case the use of penetrant aids such as DMSO facilitates the exposure of chemiluminescent-labeled oligo probes to the genome of the organism. The DNA of the organism then fluoresces under ultraviolet epi-illumination. A marvelous tutorial on probe labeling is available at www.jic.bbsrc.ac.uk/staff/pat-heslop-harrison/methods/probe.html. The method with fluorescent-labeled probes when applied for the detection of cancers or genetic effects, to human or animal genes in tissue, is called fluorescent in situ hybridization (FISH).

DEFINED OLIGOS ON MAGNETIC BEADS

Blotting and related methods have been discussed in a previous review (1). A very rapid technique employs specific oligos immobilized on Dynal© magnetic beads. The special advantage is that the beads, covalently coupled to particular oligo sequences (or antigens, antibodies, or other binding entities), can be mixed in a nonmetallic container (e.g., test tube) with a solution or suspension so as to react even with very dilute targets (organisms, especially viruses). The beads are then concentrated simply by placing a magnet against the side of the container. The original solution, less the bead-reacted targets, is then removed and the beads rinsed in situ. An oligo-magnetic bead method is outlined in (7). The recovery of mRNA (terminal polyA sequence) is with poly dT beads (8).

The basic magnetic-bead patent appears to be the property of Dynal, but Clemente Associates (formerly Quantum Magnetics, Madison, CT) produces Ni-bearing magnetic particles of 3- to 5-μm diameter for performing what they refer to as "magnetic chromatography," of cells, proteins, or both. Qiagen (Valencia, CA, and other locations) markets histidine-labeled Ni-containing agarose magnetic beads. Bangs Laboratories (Fishers, IN) also produces 2-μm diameter beads with surface caboxyl groups for the coupling of binding ligands. Seradyn (Indianapolis, IN) uses beads for DNA isolation. All of these can bear oligo probes. CPG, Inc. (Lincoln Park, NJ) offers streptavidin magnetic porous glass for the binding of biotinylated molecules for a variety of oligo-probe-related purifications of cells, noncellular particles, and macromolecules, including DNAs and RNAs.

Nonmagnetic beads of a variety of matrices, ready for coupling by various chemistries to oligos, antibodies, antigens, or what-have-you, are available for column or suspension applications from Polymer Laboratories (www.polymerlabs.com).

THE POLYMERASE CHAIN REACTION (PCR) AND THE DETECTION OF MICROBES

If a dozen copies of a pathogenic DNA virus are present per milliliter or gram in body fluid or beef tissue intended for sale, inoculation of the suspect source material into cell culture or in a test animal for analytical testing is a very lengthy, expensive process. It is unrealistic for use in the food industry or in a hospital, as are many other tests. If we seek particular viruses (e.g., rabies), the most rapid, practical, and effective means of detecting the virions is the PCR.

The general sequence of events in performing PCR is shown in Fig. 4. The primer used is an oligo specific to the organism to be detected. Commencing with the DNA recovered from one or several organisms, the multiplication of genomic material can be 10^7-to 10^8- fold in 35–90 min. By comparison, growing the virions in cell culture to equivalent numbers (to allow monoclonal antibody type analyses) usually requires 48 h or more. In cases where life hangs in the balance of an accurate analysis, those hours may be critical.

Even with bacterial or fungal pathogens, the time required to generate sufficient genomic material for analysis by PCR is 35–45 min but 24–48 h in conventional culture on an appropriate nutfrient medium. Some useful background information on PCR is provided in the Eppendorf web page (www.eppendorphsi.com/applications.html).The reader is also referred to the previous version (1), which provides details in a somewhat different manner. Primers (DNA probes needed for PCR) have been described particularly well by Steve Rozen (Whitehall Institute, MIT Center for Genomic Research, Cambridge, MA). The material he has developed is available, gratis, at www.genome.wi.mit, which appears to be difficult to recover. HIV primers are shown at www.appliedbiosystems.com/pc/catalog/pg25.html.

Reverse transcriptase PCR, (RT-PCR), runs the normal RNA to DNA sequence backward. Reverse transcriptase is an enzyme found in RNA viruses and, when added to a solution of RNA, deoxynucleotides, reverse transcriptase, etc., allows one to produce the DNA equivalent to the RNA recovered from an organism. The cDNA (DNA produced from RNA)recovered in sufficient amounts can then can be "clipped" using a well-characterized endonuclease and a cDNA "fingerprint" generated for comparison with the fingerprints of known RNA sources. Automated systems for doing this can be purchased from Qualicon, a DuPont spinoff (info@qualicon.com) or from Applied Biosystems (www.appliedbiosystems.com/ab/about/mm /microseq/microseq500/).

IMMOBILIZED OLIGOS OR GENES IN ANALYTICAL ARRAYS

As reported in the previous version of this article (1), immobilized oligos in arrays (e.g., in the wells of microtiter plates or on glass slides) are used in the detection of cancer and precursor stages (e.g., mutations), in addition to drug screening of the interactions of candidate drugs (free) with human or animal gene sequences (immobilized). A tutorial on microarray technology is presented by Virginia Tech and the Forest Biotechnology Group at the North Carolina State University (9).

To provide the reader with an appreciation of the flexibility possible with arrays, I consider the possibilities inherent in combinatorial chemistry (10). One possible approach involves the extraction of the gene of each of hundreds of bacterial species, grown as axenic (pure) cultures. In each of the wells of microtiter plates molded from polyethylene-co-acrylic acid, segments of the gene of a single bacterial species (prepared when the DNA from the cells of a particular colony are "clipped" with a known endonuclease) are added and immobilized to the bottom of the plate via the 3′-OH. A good immobilization system is the triazine method for coupling linear microbial DNA strands (Fig. 5). To each of these plates, bearing (for example) ca. 100 wells and the genomes of 100 human

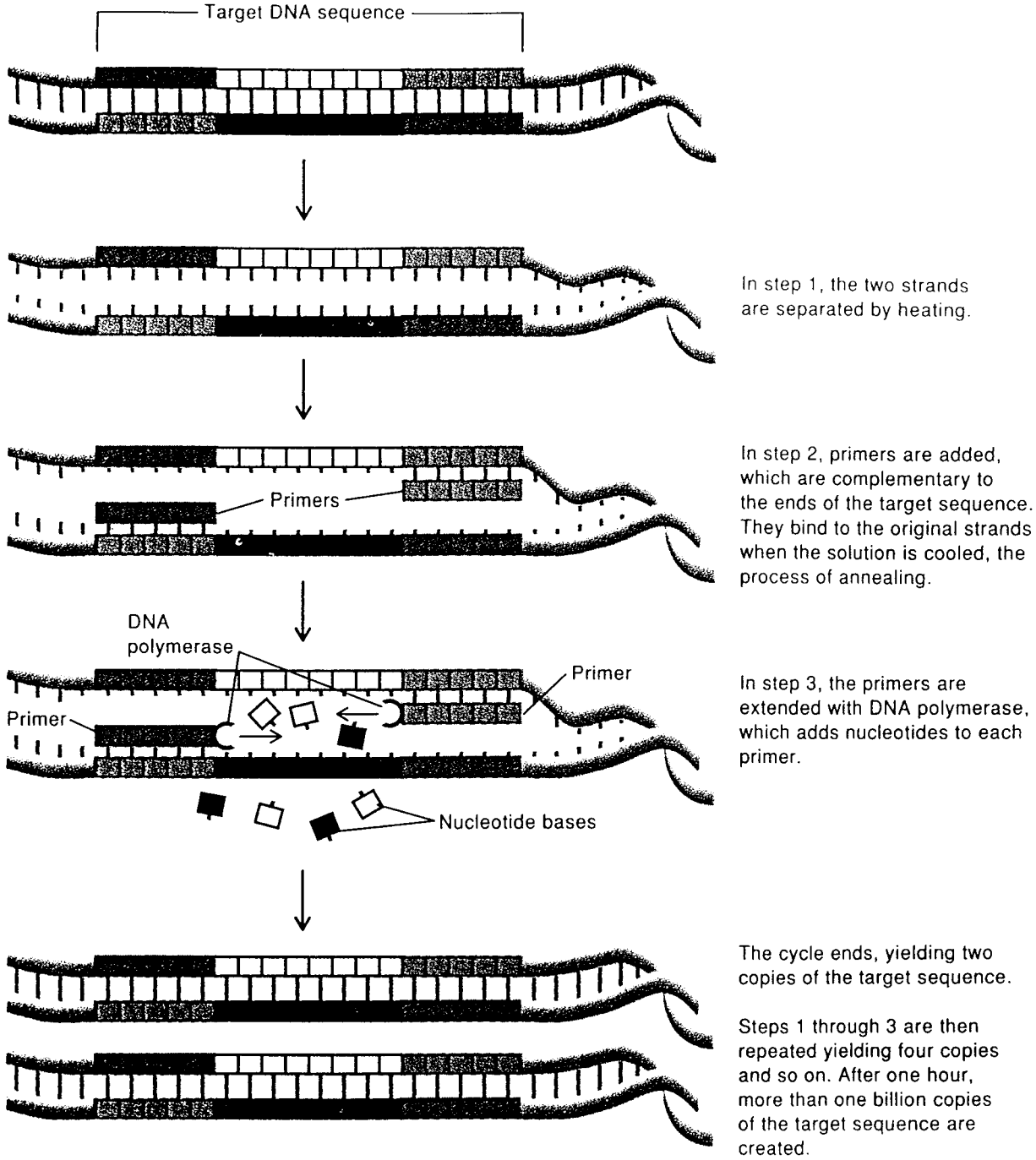

Fig. 4 Sequence of events in the PCR.

microbial pathogens, is added one of the many varieties of possible synthetic oligos intended for test as potential drugs (limited mixtures, each unpurified but reproducible), each with a low toxicity label, preferably chemiluminescent rather than radioactive.

The drugs (e.g., PNAs) and immobilized microbial genomes are allowed a reaction time, rinsed, and then examined (automatically or by eye,) for retention of the synthetic candidate drugs by segments of the genomes of the various pathogens. In this way, it is possible to screen

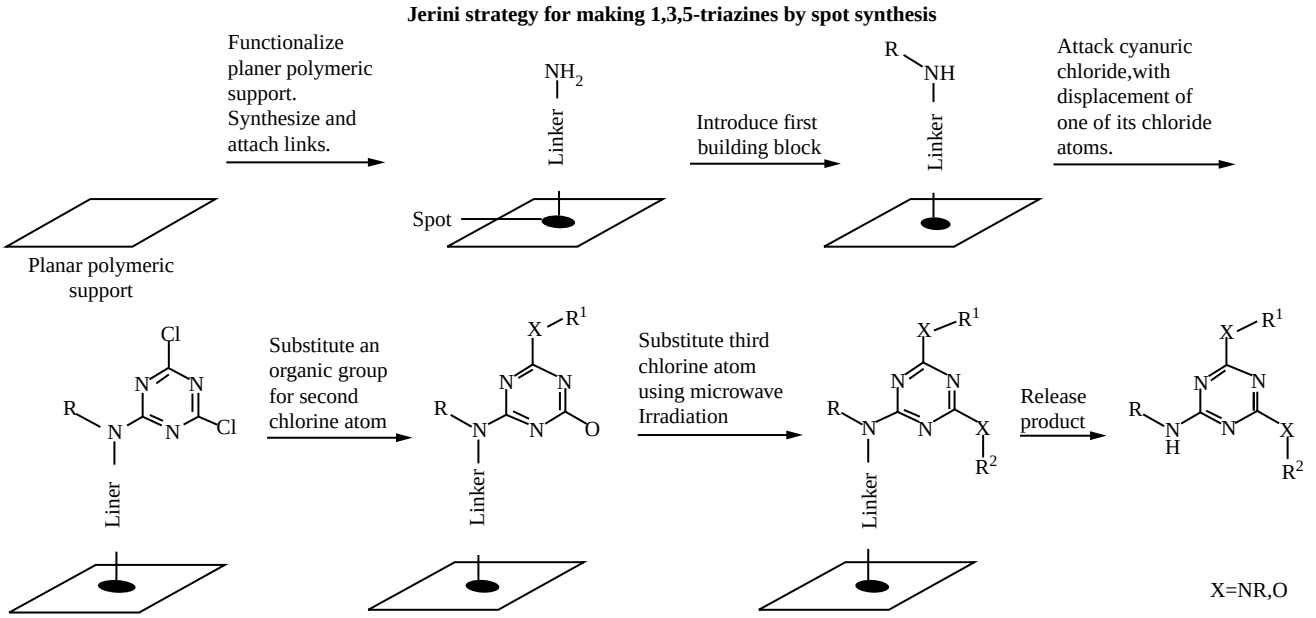

Fig. 5 Coupling microbial ssDNA or RNA or analogs via triazine to wells in a microtiter plate.

candidate oligos for specific binding to immobilized genomes of known organisms, even if gene sequences of the pathogens are unknown, and the precise sequence that binds to a given pathogen's genome is known only with modest accuracy.

In cases where this combinatorial-type approach to new product development appears promising, it can be automated, all or in part. Such systems are in widespread use in a variety of applications in the pharmaceutical industry. The example given is a variation of the method known as combinatorial chemistry.

A variation on this method has been proposed by the U.S. National Institute of Environmental Health Sciences to evaluate chemicals as potential human toxins. Human genes, immobilized on microtiter-type plates, are exposed to known and unknown toxicants, with a view to seeking similarities in gene-binding patterns (11). Origene (Rockville, MD), Qiagen (Valencia, CA, and other sites), CCS Packard (Meriden, CT), and many others may be added to the list of array-type manufacturers/distributors listed in Table 3 in Ref. 1.

SPECIFIC OLIGO PROBES AS ANTIMICROBIALS

The DNA of a cell must be copied if the cell is to reproduce. Also, certain enzymes are required for a cell to metabolize and function (as contrasted with the dormant state), and these are usually made in the cell as needed. Therefore, an antisense probe that reacts with, for example, a critical RNA in the cell, may effectively render the cell dormant. One can envision this in vitro and in vivo. But oligonucleotides are subject to degradation by nucleases which, depending on their specificity, clip a nucleotide at certain base sequences, usually rendering the oligo less functional or nonfunctional. (This is also how DNA is clipped in the process of generating "fingerprints.")

If a species-specific antisense oligo probe can be made resistant to enzymic degradation, and if it can readily enter a microbe, the probe not only identifies the organism, but also may shut down the metabolic potential of the cell (11). Antisense PNA oligos appear to meet these requirements and bind to sense with a lower dissociation constant (K_d) than does the phosphate-backbone antisense. PNA antisenses have the potential for use as very specific, low-toxicity microbicides for in vivo applications (12). The peptide analogs of DNA probes are protected by appropriate patents (13). The text of choice is from the same authors and others (14).

Boston Bioprobes is producing PNAs for oligo research, development of diagnostics, testing of foods, and the environment. Perkin-Elmer (now largely renamed Analytical Biosystems, Foster City, CA) has invested with BBI and will participate in PNA technology.

ANTISENSE PROBES AS THERAPEUTICS

The primary target of antisense probe studies is the ongoing cell proliferation in all cancers.

Cancerous cells are not functional in, for example, gas transport in the lung. Like viruses, their anthropomorphized raison d'etre is only to reproduce, not to perform a service for the organism of which they are a part. On the other hand, normal cells have a finite lifetime and must occasionally be replaced.

The toxicants used in conventional chemotherapy and radiation not only kill a substantial proportion of the cells that are reproducing rapidly but also tend to destroy stem cells in the bone marrow and circulating plasma, which gives rise to the panoply of red cells, white cells, and platelets that are essential to a survival free of dialysis machines and other elaborate supports. For this reason, patient-sourced (autologous) stem cells (CD34+), hopefully cancer-free, are recovered from the patient prior to radiation treatment or chemotherapy (or both) as "heroic" cancer therapy. The recipient of massive "chemotherapy," or radiation (or both), dies unless those healthy stem cells (removed from the patient prior to treatment) are injected intravenously (they find their way to the marrow) and replace the essential cells that chemotherapy or radiation has destroyed.

In addition, although chemotherapy and radiation may "cure" a patient, the once-healthy cells that are not killed may be mutated in critical portions of the genome. It is fairly common that patients receiving "heroic" therapy develop new tumor sets 20 years downstream of the initial therapy. Ultimately, the exposure to these unselective toxicants that saved the patient may eventually kill the patient. ("The therapy was successful, but the patient died.")

In theory, the beauty of antisense is that an antisense oligo probe does not damage all types of cells at random,

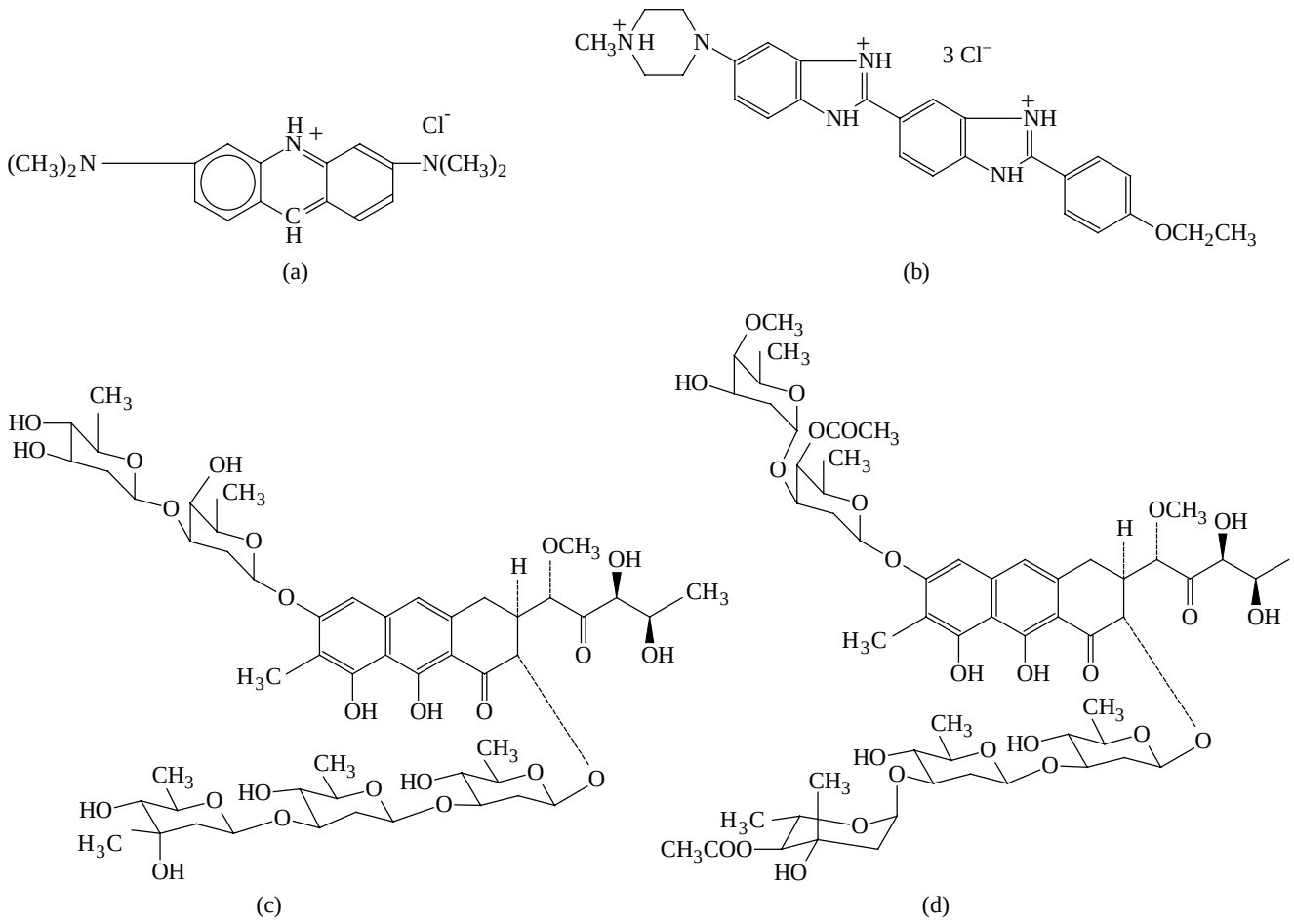

Fig. 6 Small organic molecules that interact with DNA and/or RNA and when excited with one wavelength of light, fluoresce in another. (a) acridine orange; (b) Hoechst 33342; (c) mithramycin; (d) chromamycin A3.

and does not induce mutations. Antisense shuts down the propagation of some cells, and not others. Antisense therapy is distinct from gene therapy, in which a missing or malfunctioning gene (e.g., human clotting factor VIII gene in most hemophiliacs) is placed in autologous cells and returned to the patient.

Professor Eric Wickstrom (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA) has done some excellent work in mouse models on antisense and Burkitt's lymphoma, rabies virus, and the human *cMYC* oncogene, to name a few systems. His group employs phosphorothioates, PNAs, and other variations on antisense. His web page is at www.kcc.tju.edu/staff/wickstrom and several of his recent papers (15, 16) and his book (17) should be of interest to the reader interested in antisense. His patents on the synthesis (18) and introduction of antisense into cells (19) are germane.

Some of Wickstrom's work points to the issue of antisense entry into a cell. At least one company, Gene Therapy Systems, is devoted to this (www.genetherapy-systems.com).

SMALL, NONSPECIFIC ORGANIC PROBES THAT LOCATE INTACT DNA

Some organic molecules have a high affinity (low K_d) for DNA and are stoichiometric but not specific in the manner of a complementary oligonucleotide. For example, acridine orange (AO; Fig. 6a), is planar and intercalates between the paired sets of H-bonded base pairs (A–T, G–C) in dsDNA (Fig. 2). Where AO penetrates into a cell and "reacts "with the dsDNA, the cell emits green light when excited with blue. AO also reacts with ssDNA or RNA by stacking on the charged phosphate of the backbone; ssDNA fluoresces in the red.

Benzamides such as Hoechst 33342 (Fig. 6b) bind to A–T-rich regions in the small groove of dsDNA. When sufficient dye is excited with blue light, light emission is red. The antibiotics mithramycin and chromamycin A3 (Fig. 6c and Fig. 6d) bind to G–C regions of DNA.

These small molecule probes of DNA (ds and ss) and RNA do not identify organisms, but indicate probable viability because the genome is intact. When cells in suspension or on a slide are stained and examined under a UV-epi-illuminated microscope or in a flow cytometer, with or without cell sorting, then viability can be estimated with fairly high precision and accuracy. This and related topics are developed in (7), and in portions of the molecular probes pages (www.probes.com), which the reader may find particularly useful.

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